



The antifungal plant defensin HsAFP1 from *Heuchera sanguinea* induces apoptosis in *Candida albicans*

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Plant defensins are active against plant and human pathogenic fungi (such as *Candida albicans*) and baker's yeast. However, they are non-toxic to human cells, providing a possible source for treatment of fungal infections. In this study, we characterized the mode of action of the antifungal plant defensin HsAFP1 from coral bells by screening the *Saccharomyces cerevisiae* deletion mutant library for mutants with altered HsAFP1 sensitivity and verified the obtained genetic data by biochemical assays in *S. cerevisiae* and *C. albicans*. We identified 84 genes, which when deleted conferred at least fourfold hypersensitivity or resistance to HsAFP1. A considerable part of these genes were found to be implicated in mitochondrial functionality. In line, sodium azide, which blocks the respiratory electron transport chain, antagonized HsAFP1 antifungal activity, suggesting that a functional respiratory chain is indispensable for HsAFP1 antifungal action. Since mitochondria are the main source of cellular reactive oxygen species (ROS), we investigated the ROS-inducing nature of HsAFP1. We showed that HsAFP1 treatment of *C. albicans* resulted in ROS accumulation. As ROS accumulation is one of the phenotypic markers of apoptosis in yeast, we could further demonstrate that HsAFP1 induced apoptosis in *C. albicans*. These data provide novel mechanistic insights in the mode of action of a plant defensin.

Keywords: plant defensin, *Candida albicans*, *Saccharomyces cerevisiae*, mitochondria, apoptosis, mode of action

INTRODUCTION

Plant defensins are small, basic, cysteine-rich peptides that possess antifungal and in some cases also antibacterial activity (Lay and Anderson, 2005; Aerts et al., 2008). They are postulated to be part of the innate immune system of plants and protect them against invading fungal and bacterial pathogens. Plant defensins are not only active against phytopathogenic fungi, but also against baker's yeast *Saccharomyces cerevisiae* and human pathogenic fungi (such as *Candida albicans*). *C. albicans* is an opportunistic human pathogen that can cause superficial and invasive infections in immunocompromised patients (Mavor et al., 2005). Plant defensins are non-toxic to human and plant cells (Thevissen et al., 2007a; Tavares et al., 2008), highlighting their therapeutic potential as novel antimicrobials. In this regard, radish defensin RsAFP2 (Terras et al., 1992) was recently proven to be prophylactically effective against murine candidiasis, and this at least to the same extent as the antimicrobial fluconazole (Tavares et al., 2008).

HsAFP1 is a plant defensin, isolated from seeds of coral bells (*Heuchera sanguinea*; Osborn et al., 1995). HsAFP1 inhibits a wide range of fungi as well as the pathogenic yeast/fungal species *C. albicans*, *C. krusei*, and *Aspergillus flavus* (Thevissen et al., 2007a). We previously demonstrated that HsAFP1 specifically interacts with high affinity binding sites on the membrane of susceptible fungi and yeast species (Thevissen et al., 1997), and permeabilizes susceptible fungal/yeast cells, resulting in cell growth arrest (Thevissen et al., 1999). In contrast to HsAFP1, RsAFP2, pea defensin PsD1 (Almeida et al., 2000), and dahlia

defensin DmAMP1 (Osborn et al., 1995) specifically interact with sphingolipids in the fungal envelope, being glucosylceramides (GlcCer) or mannosyl diinositolphosphoryl ceramides [M(IP)2C; Thevissen et al., 2000, 2003, 2004; de Medeiros et al., 2010]. GlcCer were also found indispensable for the antifungal activity of *Medicago* defensin MsDef1 (Ramamoorthy et al., 2007). The antifungal activity of HsAFP1 does not rely on the interaction with these sphingolipids since yeast mutants lacking GlcCer or M(IP)2C are as sensitive to HsAFP1 as their corresponding wild type (WT; unpublished data). The *in vitro* frequency of occurrence of spontaneous RsAFP2-resistant *C. albicans* mutants is 5–10 times higher than that of DmAMP1-resistant mutants and at least 100 times higher than that of HsAFP1-resistant *C. albicans* mutants (Thevissen et al., 2007a). Possibly, HsAFP1 interacts with essential fungal plasma membrane structures, resulting in low *in vitro* frequency of occurrence of resistant *C. albicans* mutants. Therefore, in view of reducing the risks of rapid emergence of resistant pathogens, HsAFP1 may offer advantages over RsAFP2 and DmAMP1 as novel antifungal agents.

In this study, we screened the haploid set of *S. cerevisiae* deletion mutants in non-essential genes for both hypersensitivity and resistance to HsAFP1 in order to get further insight in the mode of action of HsAFP1. Based on these genetic data, we could demonstrate the involvement of mitochondrial function in HsAFP1 antifungal action using the respiration inhibitor sodium azide and by investigating the accumulation of reactive oxygen species (ROS) in susceptible yeast species upon HsAFP1 treatment. Since

mitochondrial function and the accumulation of endogenous ROS or both linked with apoptosis in yeast, we assessed the apoptosis-inducing nature of HsAFP1 in *C. albicans*.

MATERIALS AND METHODS

MATERIALS AND MICROORGANISMS

Yeast strains used in this study are *C. albicans* strain CAI4 (Fonzi and Irwin, 1993), *S. cerevisiae* strains W0303-1a, BY4741, and the BY4741-derived deletion mutant library (Invitrogen, Carlsbad, CA, USA). Yeast nutrient media used are YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose); PDB/YPD (19.2 g/l potato dextrose broth (Difco), 2 g/l yeast extract, 4 g/l peptone, 4 g/l glucose; adjusted to pH 7.0 with 50 mM HEPES); and SC (0.8 g/l CSM, complete amino acid supplement mixture, Bio 101 Systems; 6.5 g/l YNB, yeast nitrogen base; 20 g/l glucose). HsAFP1 was purified as described previously (Osborn et al., 1995). *H. sanguinea* seeds were kindly provided by Kieft Seeds (Venhuizen, The Netherlands). If not mentioned otherwise, chemicals were purchased from Sigma (St. Louis, MO, USA).

SCREENING OF A *S. CEREVISIAE* DELETION MUTANT LIBRARY FOR ALTERED HsAFP1 SENSITIVITY

To this end, the minimal inhibitory concentration (MIC) of HsAFP1 for the individual deletion mutants was determined in PDB/YPD and compared with the MIC of HsAFP1 for WT (Thevisen et al., 2007b). The HsAFP1-hypersensitivity (HSFs) or resistance factors (RFs) were calculated as MIC(WT)/MIC(mutant) or MIC(mutant)/MIC(WT), respectively. Strains that were at least fourfold more resistant or hypersensitive to HsAFP1 were retested.

ANTIFUNGAL ACTIVITY ASSAY

Exponentially growing *S. cerevisiae* (W303-1a) in YPD ($OD_{600} = 2.0$) were incubated with 20 μ g/ml HsAFP1 in the presence or absence of 0.005% sodium azide in PDB/YPD medium as described previously (Aerts et al., 2009a).

YEAST APOPTOSIS ASSAYS

Exponentially growing *C. albicans* cultures (SC, $OD_{600} = 2.0$) were incubated with 5 μ g/ml HsAFP1 or water (control) in PDB/YPD during 2 h 30 min at 30°C. Survival was determined by performing plating assays in which colony formation of 500 cells on YPD agar plates was analyzed. Apoptotic markers, including ROS levels, phosphatidylserine (PS) externalization, and DNA fragmentation of yeast cultures ($n = 500$ cells per measurement), were visualized as described previously (Aerts et al., 2009a).

RESULTS

SCREENING OF A YEAST DELETION MUTANT LIBRARY FOR HYPERSENSITIVITY AND RESISTANCE TOWARD HsAFP1 REVEALS A CRUCIAL ROLE FOR MITOCHONDRIA IN THE MODE OF ACTION OF HsAFP1

To identify genes that may contribute to HsAFP1 tolerance or sensitivity, we screened a *S. cerevisiae* deletion mutant library (consisting of 4385 deletion mutants) for hypersensitivity and resistance toward HsAFP1. To this end, we determined the MIC resulting in 100% growth inhibition of HsAFP1 for all individual yeast knock-out mutants and WT yeast using twofold dilution

series of HsAFP1 in liquid PDB/YPD medium. We identified 13 HsAFP1-resistant and 71 HsAFP1-hypersensitive mutants, which were at least fourfold more resistant or sensitive toward HsAFP1 as compared to WT (MIC = 20 μ g/ml), and the corresponding HsAFP1-sensitivity and tolerance genes, respectively. Following major functional families of HsAFP1-sensitivity or -tolerance genes could be distinguished (according to www.yeastgenome.org), encoding gene products involved in: (i) vacuolar acidification and protein sorting/vesicular transport, (ii) gene expression/DNA repair, (iii) mitochondrial function, (iv) cytoskeletal organization and cytokinesis, (v) cell wall biosynthesis and maintenance, and (vi) stress response signaling (Table 1). Interestingly, 30% of the HsAFP1-sensitivity genes encode proteins that have a mitochondrial function, suggesting an important role for mitochondria in HsAFP1-mediated cell death. Note that the portion of disruptants in non-essential genes, which encode proteins involved in mitochondrial function, in the yeast library is approximately 7% (Dimmer et al., 2002), indicating a fourfold enrichment of genes important for proper mitochondrial function within the HsAFP1-sensitivity genes. Mitochondrial function is known to be important for the induction of apoptosis in yeast (Eisenberg et al., 2007; Fröhlich et al., 2007). In addition, among the genes subdivided in the groups (ii), (iii), and (vi), we found eight HsAFP1-tolerance or -sensitivity genes implicated in apoptosis in yeast (being *DHH1*, *LSM1*, *LSM6*, *RIP1*, *SIR4*, *HDA2*, *KAP123*, and *SCP160*). Therefore, in this study, we further investigated the involvement of functional mitochondria in the antifungal action of HsAFP1 and whether the induction of apoptosis is involved in this process.

BLOCKING MITOCHONDRIAL FUNCTION BY RESPIRATION INHIBITOR SODIUM AZIDE ANTAGONIZES HsAFP1 ANTIFUNGAL CAPACITY

Several of the identified HsAFP1-tolerance or -sensitivity genes with a mitochondrial function are directly implicated in respiratory growth, i.e., *RIP1*, *YDR115w*, *CEM1*, *ATP12*, *COX17*, and *MRPL35*. Therefore, we investigated the effect of the respiration inhibitor sodium azide on HsAFP1 antifungal action. Sodium azide blocks the electron flow at complex IV (cytochrome *c* oxidase) of the respiratory electron transport chain thereby preventing ATP production. We previously demonstrated that treatment of susceptible fungi with 50–100 μ g/ml plant defensins results in a specific membrane permeabilization, whereas treatment with lower doses, i.e., 5–20 μ g/ml, results in target-mediated membrane permeabilization (Thevisen et al., 1999). Therefore, in this study, we used rather low HsAFP1 concentrations, i.e., 5–20 μ g/ml.

To assess the effect of sodium azide on HsAFP1 antifungal action, we determined survival of *S. cerevisiae* cultures, incubated with 20 μ g/ml HsAFP1 or water in the presence or absence of 0.005% azide for different incubation times (Figure 1).

Incubation of the yeast culture with 20 μ g/ml HsAFP1 for 0, 4, or 8 h resulted in 100, 1, and 0.0002% survival of the culture relative to the control (water) treatment, whereas co-incubation with 0.005% sodium azide resulted in 100% survival of these cultures. Hence, sodium azide antagonizes HsAFP1 antifungal action up till 8 h of incubation. These results suggest that a functional respiratory chain is indispensable for HsAFP1 antifungal action.

Table 1 | HsAFP1-tolerance and -sensitivity genes.

ORF	Gene	Description function gene product	HSP
A. HsAFP1-TOLERANCE GENES (CORRESPONDING DELETION MUTANTS ARE HYPERSENSITIVE TO HSAFP1)			
(I) VACUOLAR ACIDIFICATION AND PROTEIN SORTING/VESICULAR TRANSPORT			
YGR167W	<i>CLC1</i>	Clathrin light chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis	8
YEL027W	<i>CUP5</i>	Proteolipid subunit of the vacuolar H ⁺ -ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis	4
YDR017C	<i>KCS1</i>	Inositol hexaphosphate kinase, phosphorylates inositol hexakisphosphate (InsP6) to diphosphoinositol polyphosphates, required for proper vacuole morphology and involved in salt stress response	64
YDR320C	<i>SWA2</i>	Auxilin-like protein involved in vesicular transport; clathrin-binding protein required for uncoating of clathrin-coated vesicles	32
YDR126W	<i>SWF1</i>	Palmitoyltransferase that acts on the SNAREs Snc1p, Syn8p, Tlg1p, and likely on all SNAREs; member of a family of putative palmitoyltransferases containing an Asp-His-His-Cys-cysteine rich (DHHC-CRD) domain; may have a role in vacuole fusion	4
YBR127C	<i>VMA2</i>	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H ⁺ -ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system	8
YLR373C	<i>VID22</i>	Glycosylated integral membrane protein localized to the plasma membrane; plays a role in fructose-1,6-bisphosphatase (FBPase) degradation; involved in FBPase transport from the cytosol to Vid (vacuole import and degradation) vesicles	4
YKL080W	<i>VMA5</i>	Subunit C of the eight-subunit V1 peripheral membrane domain of vacuolar H ⁺ -ATPase	4
YLR447C	<i>VMA6</i>	Subunit D of the five-subunit V0 integral membrane domain of vacuolar H ⁺ -ATPase	8
YGR020C	<i>VMA7</i>	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H ⁺ -ATPase	32
YEL051W	<i>VMA8</i>	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H ⁺ -ATPase	4
YHR039C-A	<i>VMA10</i>	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H ⁺ -ATPase	4
YHR060W	<i>VMA22</i>	Integral ER membrane protein that is required for assembly of vacuolar H ⁺ -ATPase function	4
YKL119C	<i>VPH2</i>	Integral ER membrane protein that is required for assembly of vacuolar H ⁺ -ATPase function	64
YKR020W	<i>VPS51</i>	Component of the Golgi-associated retrograde protein (GARP) complex, Vps51p-Vps52p-Vps53p-Vps54p, which is required for the recycling of proteins from endosomes to the late Golgi; links the (VFT/GARP) complex to the SNARE Tlg1p	8
YJL029C	<i>VPS53</i>	Component of the Golgi-associated retrograde protein (GARP) complex	16
YPR139C	<i>VPS66</i>	Cytoplasmic protein of unknown function involved in vacuolar protein sorting	4
(II) GENE EXPRESSION/DNA REPAIR			
YDR448W	<i>ADA2</i>	Transcription coactivator, component of the ADA and SAGA transcriptional adaptor/histone acetyltransferase (HAT) complexes	8
YLR226W	<i>BUR2</i>	Cyclin for the Sgv1p (Bur1p) protein kinase; Sgv1p and Bur2p comprise a CDK-cyclin complex involved in transcriptional regulation through its phosphorylation of the carboxy-terminal domain of the largest subunit of RNA polymerase II	8
YKL054C	<i>DEF1</i>	RNAPII degradation factor, forms a complex with Rad26p in chromatin, enables ubiquitination and proteolysis of RNAPII present in an elongation complex	256
YFL001W	<i>DEG1</i>	Non-essential tRNA:pseudouridine synthase, introduces pseudouridines at positions 38/39 in tRNA, important for maintenance of translation efficiency and cell growth	4
YDL160C	<i>DHH1</i>	Cytoplasmic DExD/H-box helicase, stimulates mRNA decapping, coordinates distinct steps in mRNA function and decay, interacts with both the decapping and deadenylase complexes, may have a role in mRNA export and translation	32
YNL133C	<i>FYV6</i>	Protein of unknown function, required for survival upon exposure to K1 killer toxin; proposed to regulate double-strand break repair via non-homologous end-joining	4
YDR295C	<i>HDA2</i>	Subunit of a possibly tetrameric trichostatin A-sensitive class II histone deacetylase complex containing an Hda1p homodimer and an Hda2p-Hda3p heterodimer; involved in telomere maintenance	32
YER110C	<i>KAP123</i>	Karyopherin beta, mediates nuclear import of ribosomal proteins prior to assembly into ribosomes and import of histones H3 and H4	4
YGL173C	<i>KEM1</i>	Evolutionarily-conserved 5'-3' exonuclease component of cytoplasmic processing (P) bodies involved in mRNA decay; plays a role in microtubule-mediated processes, filamentous growth, ribosomal RNA maturation, and telomere maintenance	16
YFR001W	<i>LOC1</i>	Nuclear protein involved in asymmetric localization of ASH1 mRNA; binds double-stranded RNA <i>in vitro</i> ; constituent of 66S pre-ribosomal particles	16
YJL124C	<i>LSM1</i>	Lsm (Like Sm) protein; forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of cytoplasmic mRNAs	4

(Continued)

Table 1 | Continued

ORF	Gene	Description function gene product	HSP
YDR378C	<i>LSM6</i>	part of cytoplasmic Lsm1p complex involved in mRNA decay; and nuclear Lsm8p complex part of U6 snRNP and possibly involved in processing tRNA, snoRNA, and rRNA	16
YLR320W	<i>MMS22</i>	Protein involved in resistance to ionizing radiation; acts with Mms1p in a repair pathway that may be involved in resolving replication intermediates or preventing the damage caused by blocked replication forks	4
YKL074C	<i>MUD2</i>	Protein involved in early pre-mRNA splicing; component of the pre-mRNA-U1 snRNP complex, the commitment complex; interacts with Msl5p/BBP splicing factor and Sub2p	32
YJL140W	<i>RPB4</i>	RNA polymerase II subunit B32; involved in export of mRNA to cytoplasm under stress conditions; involved in telomere maintenance	64
YGR056W	<i>RSC1</i>	One of 15 subunits of the “Remodel the Structure of Chromatin” (RSC) complex; required for expression of mid-late sporulation-specific genes	16
YHL025W	<i>SNF6</i>	One of 11 subunits of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf5p	4
YGR063C	<i>SPT4</i>	Protein that forms a complex with Spt5p and mediates both activation and inhibition of transcription elongation, and plays a role in pre-mRNA processing; kinetochore function and gene silencing	32
YBR081C	<i>SPT7</i>	Subunit of the SAGA transcriptional regulatory complex, involved in proper assembly of the complex; also present as a C-terminally truncated form in the SLIK/SALSA transcriptional regulatory complex	4
YJL127C	<i>SPT10</i>	Putative histone acetylase, required for transcriptional regulation at core promoters, functions at or near the TATA box	32
YDR092W	<i>UBC13</i>	Ubiquitin-conjugating enzyme involved in the error-free DNA postreplication repair pathway; interacts with Mms2p to assemble ubiquitin chains at the Ub Lys-63 residue	4
YDR207C	<i>UME6</i>	Key transcriptional regulator of early meiotic genes, binds URS1 upstream regulatory sequence, couples metabolic responses to nutritional cues with initiation and progression of meiosis, forms complex with Ime1p, and also with Sin3p–Rpd3p	4
(III) MITOCHONDRIAL FUNCTION			
YDR226W	<i>ADK1</i>	Adenylate kinase, required for purine metabolism; localized to the cytoplasm and the mitochondria	128
YJL180C	<i>ATP12</i>	Molecular chaperone, required for the assembly of alpha and beta subunits into the F1 sector of mitochondrial F1F0 ATP synthase	32
YER061C	<i>CEM1</i>	Mitochondrial beta-keto-acyl synthase with possible role in fatty acid synthesis; required for mitochondrial respiration	32
YJR118C	<i>ILM1</i>	Protein of unknown function; may be involved in mitochondrial DNA maintenance; required for slowed DNA synthesis-induced filamentous growth	4
YCR028C-A	<i>RIM1</i>	Single-stranded DNA-binding protein essential for mitochondrial genome maintenance; involved in mitochondrial DNA replication	4
YEL024W	<i>RIP1</i>	Ubiquinol-cytochrome-c reductase, a Rieske iron-sulfur protein of the mitochondrial cytochrome bc1 complex; transfers electrons from ubiquinol to cytochrome c1 during respiration	4
YDR115W		Putative mitochondrial ribosomal protein of the large subunit, required for respiratory growth, as are most mitochondrial ribosomal proteins	64
(IV) CYTOSKELETAL ORGANIZATION AND CYTOKINESIS			
YFL023W	<i>BUD27</i>	Protein involved in bud-site selection, nutrient signaling, and gene expression controlled by the TOR kinase	32
YER014C-A	<i>BUD25</i>	Protein involved in bud-site selection	4
YCR002C	<i>CDC10</i>	Component of the septin ring of the mother-bud neck that is required for cytokinesis	4
YMR032W	<i>HOF1</i>	Bud neck-localized, SH3 domain-containing protein required for cytokinesis; regulates actomyosin ring dynamics and septin localization; interacts with the formins, Bni1p and Bnr1p, and with Cyk3p, Vrp1p, and Bni5p	4
YDR435C	<i>PPM1</i>	Carboxyl methyl transferase, methylates the C terminus of the protein phosphatase 2A catalytic subunit (Pph21p or Pph22p), which is important for complex formation with regulatory subunits	4
YLR337C	<i>VRP1</i>	Proline-rich, actin-associated protein involved in cytoskeletal organization and cytokinesis	8
(V) CELL WALL BIOSYNTHESIS AND MAINTENANCE			
YHR142W	<i>CHS7</i>	Protein of unknown function, involved in chitin biosynthesis by regulating Chs3p export from the ER	4
YMR307W	<i>GAS1</i>	Beta-1,3-glucanosyltransferase, required for cell wall assembly; localizes to the cell surface via a glycosylphosphatidylinositol (GPI) anchor	16
YJL183W	<i>MNN11</i>	Subunit of a early Golgi compartment (Sed5 compartment), mannosyltransferase complex that also contains Anp1p, Mnn9p, Mnn10p, and Hoc1p, and mediates elongation of the polysaccharide mannan backbone	8
YML115C	<i>VAN1</i>	Component of the mannan polymerase I; forms a complex with Mnn9p, which is involved in mannan synthesis	4
YDR484W	<i>VPS52</i>	Component of the GARP (Golgi-associated retrograde protein) complex, Vps51p–Vps52p–Vps53p–Vps54p, which is required for the recycling of proteins from endosomes to the late Golgi; involved in localization of actin and chitin	32

(Continued)

Table 1 | Continued

ORF	Gene	Description function gene product	HSP ^a
(VI) STRESS RESPONSE SIGNALING			
YAL021C	<i>CCR4</i>	Carbon catabolite repression. Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening	8
YLR418C	<i>CDC73</i>	Substituent of Paf1 complex with RNA polymerase II, Paf1p, Hpr1p, Ctr9, Leo1, Rtf1, and Ccr4p, modification of some histones, and telomere maintenance	4
YGL244W	<i>RTF1</i>	Subunit of the RNA polymerase II-associated Paf1 complex; directly or indirectly regulates DNA-binding properties of Spt15p and relative activities of different TATA elements; involved in telomere maintenance	4
YDL006W	<i>PTC1</i>	Type 2C protein phosphatase (PP2C); inactivates the osmosensing MAPK cascade by dephosphorylating Hog1p	4
YJL080C	<i>SCP160</i>	Essential RNA-binding G protein effector of mating response pathway, predominantly associated with nuclear envelope and ER, interacts in mRNA-dependent manner with translating ribosomes via multiple KH domains	4
YER111C	<i>SWI4</i>	DNA binding component of the SBF complex (Swi4p–Swi6p), a transcriptional activator that in concert with MBF (Mbp1–Swi6p) regulates late G1-specific transcription of targets including cyclins and genes required for DNA synthesis and repair	8
(VII) VARIA/UNKNOWN			
YJR105W	<i>ADO1</i>	Adenosine kinase, required for the utilization of S-adenosylmethionine (AdoMet)	64
YLR242C	<i>ARV1</i>	Protein required for normal intracellular sterol distribution and for sphingolipid metabolism	32
YPL055C	<i>LGE1</i>	Protein of unknown function	4
YGL115W	<i>SNF4</i>	Protein kinase activator found in a complex containing Snf1p and members of the Sip1p/Sip2p/Gal83p family; activates the Snf1p protein kinase; involved in expression of glucose-repressed genes, sporulation, and peroxisome biogenesis	8
YDL001W	<i>RMD1</i>	Cytoplasmic protein required for sporulation	64
YGL160W		Putative protein of unknown function with sequence similarity to iron/copper reductases (FRE1-8), possibly involved in iron homeostasis	4
YGR131W		Hypothetical protein	64
ORF	Gene	Description gene product	RF ^b
B. HsAFP1-SENSITIVITY GENES (CORRESPONDING DELETION MUTANTS ARE RESISTANT TO HsAFP1)			
(I) VACUOLAR ACIDIFICATION AND PROTEIN SORTING/VESICULAR TRANSPORT			
YFL025C	<i>BST1</i>	GPI inositol deacylase of the ER that negatively regulates COPII vesicle formation, prevents production of vesicles with defective subunits, required for proper discrimination between resident ER proteins and Golgi-bound cargo molecules	32
YML067C	<i>ERV41</i>	Protein localized to COPII-coated vesicles, forms a complex with Erv46p; involved in the membrane fusion stage of transport	4
YIL076W	<i>SEC28</i>	Epsilon-COP subunit of the coatomer; regulates retrograde Golgi-to-ER protein traffic; stabilizes Cop1p, the alpha-COP and the coatomer complex	16
YLL040C	<i>VPS13</i>	Protein of unknown function; heterooligomeric or homooligomeric complex; peripherally associated with membranes; involved in sporulation, vacuolar protein sorting, and protein–Golgi retention	4
(II) GENE EXPRESSION/DNA REPAIR			
YOR033C	<i>EXO1</i>	5'–3' exonuclease and flap-endonuclease involved in recombination, double-strand break repair and DNA mismatch repair; member of the Rad2p nuclease family, with conserved N and I nuclease domains	4
YDR227W	<i>SIR4</i>	Silent information regulator that is involved in assembly of silent chromatin domains at telomeres and the silent mating-type loci; some alleles of SIR4 prolong lifespan	4
(III) MITOCHONDRIAL FUNCTION			
YLL009C	<i>COX17</i>	Copper metallochaperone that transfers copper to Sco1p and Cox11p for eventual delivery to cytochrome c oxidase	4
YDR322W	<i>MRPL35</i>	Mitochondrial ribosomal protein of the large subunit	4
YNL122C		Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to mitochondria	8
YCR024C	<i>SLM5</i>	Mitochondrial asparaginyl-tRNA synthetase	4
(IV) VARIA/UNKNOWN			
YNL145W	<i>MFA2</i>	Mating pheromone a-factor; interacts with alpha cells to induce cell cycle arrest and other responses leading to mating	4
YNL057W		Hypothetical protein	4
YNL143C		Hypothetical protein	8

^aHSP, HsAFP1-hypersensitivity factor [MIC(BY4741)/MIC(deletion mutant)].

^bRF, HsAFP1-resistance factor [MIC(deletion mutant)/MIC(BY4741)].

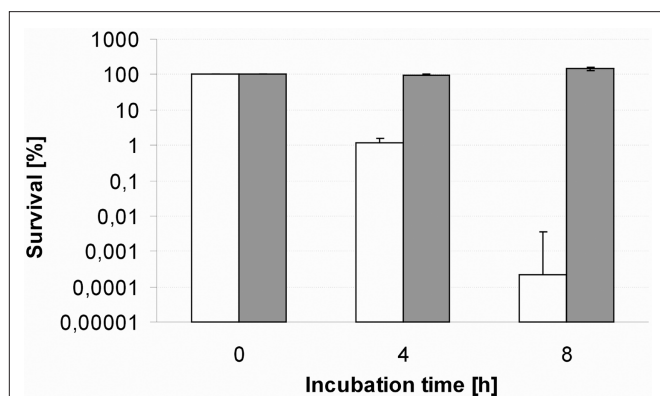


FIGURE 1 | Effect of the respiration inhibitor sodium azide on HsAFP1 antifungal action. Exponentially growing *S. cerevisiae* cultures were suspended in PDB/YPD and incubated with 20 μ g/ml HsAFP1 in the presence (black bars) or absence (white bars) of 0.005% azide for 0, 4, and 8 h of incubation at 30°C. Viability was analyzed by counting the number of CFUs/ml on YPD agar plates. Percentage survival was calculated as the ratio of the CFUs/ml after HsAFP1 treatment to the CFUs/ml of the corresponding control (water) treatment. Data represent mean \pm SEM. This figure is a representative of three independent experiments.

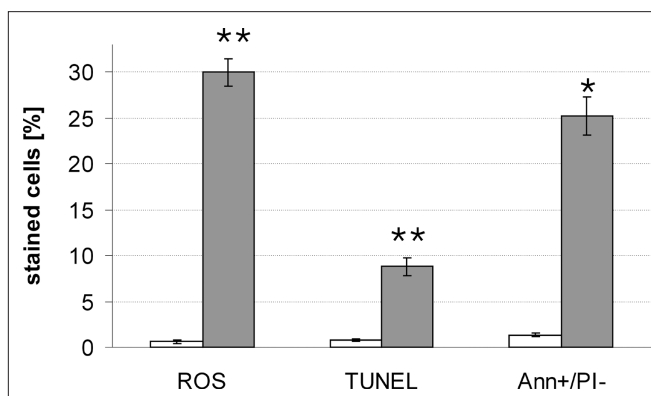


FIGURE 2 | HsAFP1 induces apoptosis in *C. albicans*. Exponentially growing *C. albicans* cultures were treated with 5 μ g/ml HsAFP1 or water for 2 h 30 min. HsAFP1-treated cells (gray bars) and control cells (white bars) were assayed for ROS accumulation (via DHE staining), DNA fragmentation (via TUNEL staining), and phosphatidylserine externalization and membrane integrity via annexinV/propidium iodide co-staining. In each experiment, 500 cells were evaluated using fluorescence microscopy (100% represents the number of cells, i.e., 500). Values are the mean of triplicate measurements. Data represent mean \pm SEM. * $p < 0.05$; ** $p < 0.01$.

HsAFP1 INDUCES ENDOGENOUS ROS IN SUSCEPTIBLE YEAST

As mentioned above, the considerable part of mitochondrial related HsAFP1-sensitivity genes suggest a role for mitochondria in the HsAFP1 antifungal activity. Since mitochondria, and more specifically complexes I and III from the respiratory chain, are the main source of cellular ROS (Batandier et al., 2002), we further assessed a putative effect of HsAFP1 on endogenous ROS levels in susceptible yeast species.

We could demonstrate that the levels of endogenous ROS increased upon HsAFP1 treatment in both *S. cerevisiae* (data not shown) and *C. albicans* (Figure 2).

HsAFP1 INDUCES APOPTOSIS IN *C. ALBICANS*

Mitochondrial function and the accumulation of endogenous ROS are both linked with apoptosis in yeast. In addition, among the genes subdivided in the functional families (ii), (iii), and (vi), we found several HsAFP1-tolerance and -sensitivity genes implicated in apoptosis in yeast, being *DHH1*, *LSM1*, *LSM6* (Mazzoni et al., 2003), *RIP1* (Belhocine et al., 2004), *SIR4* (Orlandi et al., 2004), *HDA2* (Ahn et al., 2006), *KAP123* (Büttner et al., 2007), and *SCP160* (Magherini et al., 2007). In order to clarify if the HsAFP1-induced cell death is of apoptotic nature, we assessed DNA fragmentation [visualized via the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay] and, both PS translocation from the inner leaflet to the extracellular side of the plasma membrane and loss of membrane integrity (visualized via co-staining with FITC-labeled annexin V and propidium iodide) of *C. albicans* cultures treated with 5 μ g/ml HsAFP1. HsAFP1-treated cultures were characterized by increased ROS levels ($30.0 \pm 1.5\%$) as compared to control cultures ($0.6 \pm 0.2\%$), increased DNA fragmentation levels ($8.8 \pm 1.0\%$) as compared to control treatment cultures ($0.8 \pm 0.1\%$), and by an excess of annexin V positive/PI negative cells ($25.2 \pm 2.1\%$) as compared to control cultures ($1.36 \pm 0.2\%$; Figure 2). The level of annexin V negative/positive, PI positive cells

that represent late apoptotic and necrotic cells was $23.5 \pm 3.1\%$ after HsAFP1 treatment. In conclusion, HsAFP1-induced cell death in *C. albicans* is accompanied by the occurrence of typical phenotypical markers of apoptosis, thus, pointing to a HsAFP1 antifungal mechanism that involves the induction of the apoptotic machinery in *C. albicans*.

DISCUSSION

In this study, we screened a *S. cerevisiae* deletion mutant library for altered sensitivity toward HsAFP1. In this way, we identified 71 HsAFP1-tolerance genes and 13 HsAFP1-sensitivity genes (Table 1), which we subdivided in functional families. Additional biochemical tests revealed that (i) HsAFP1 antifungal action requires a properly working yeast respiratory chain and that (ii) HsAFP1 induces apoptosis in *C. albicans*.

In mitochondria, respiration takes place supplying the cell with energy (ATP), meanwhile however leaking huge amounts of ROS. Indeed, the vast majority of cellular ROS (estimated at approximately 90%) can be tracked back to the mitochondria where sites I and III are the main sites of production (Batandier et al., 2002). ROS, such as hydrogen peroxide and hydroxyl radicals are produced as byproducts of aerobic respiration and cause damage to proteins, lipids, and DNA, resulting in mutation and loss of viability. In this study, we show that the respiration inhibitor sodium azide antagonizes HsAFP1 antifungal action, pointing to a functional respiratory chain as a prerequisite for HsAFP1 antifungal action. In line with this observation, we previously isolated the *S. cerevisiae* transposon mutant HsTnII, which is resistant toward HsAFP1 (data not shown) and characterized by respiration deficiency (Aerts et al., 2009b). All these data point to the importance of functional mitochondria and a functional respiratory chain for HsAFP1 antifungal action. Apparently, treatment of *C. albicans* cells with sodium azide also results in a decreased susceptibility to human β -defensin 2 (HBD2) and HBD3 (Vylkova et al., 2007),

pointing toward HBD2- and HBD3-induced energy dependent *C. albicans* killing, as is the case for HsAFP1. However, putative induction of apoptosis by human defensins has never been reported.

The purpose of apoptosis in multicellular organisms is obvious: single cells die for the benefit of the whole organism (e.g., to eliminate dangerous, superfluous, or damaged cells). The phenomenon of yeast cells undergoing apoptosis has long been controversial, in part because of doubts of whether cell suicide could constitute an evolutionary advantage for unicellular organisms. Studies have now described yeast apoptosis during mating, aging, or exposure to killer toxins (Büttner et al., 2006). Pheromone signaling leads to the apoptotic death of cells that fail to mate, therefore depleting the population of haploid cells and favoring the survival of diploid cells that increase genetic diversity through meiotic recombination. The early death of old and damaged cells during aging and starvation enhances the chances of the rest of the population to survive and to sporulate, thus increasing the probability that the clone will survive. Apoptosis can also be induced by competing yeast strains that produce toxins in a tribal war. The death of infertile, old, or damaged yeast cells may therefore ensure the survival of a colony of yeast cells and introduces the concept of an altruistic aging and death program. In this context, we could also understand why it may be beneficial for a yeast cell in culture to undergo apoptosis in response to an antifungal defensin.

It is well documented that mitochondrial function is linked with apoptosis in *S. cerevisiae*. Furthermore, an excess of endogenous ROS levels is one of the phenotypical markers of apoptosis in both *S. cerevisiae* (Madeo et al., 1999) and in *C. albicans* (Phillips et al., 2003). In this study, we demonstrate that HsAFP1 induces apoptotic cell death in *C. albicans*. We show that, besides ROS accumulation, HsAFP1-treated *C. albicans* cultures exhibit other key markers of apoptosis, including exposure of PS to the outer leaflet of the plasma membrane and DNA fragmentation. All these data point to the induction of mitochondrion-dependent apoptosis by HsAFP1 in susceptible yeast. These data are in line with recently obtained results indicating that also RsAFP2, a plant defensin from radish (Terras et al., 1992), induces apoptosis in *C. albicans* (Aerts et al., 2009a). In contrast to HsAFP1 for which the fungal membrane target has not yet been identified, RsAFP2 has been shown to interact with GlcCer in the membrane of susceptible fungi (Thevisen et al., 2004) and cause membrane permeabilization and fungal cell death (Thevisen et al., 1999). Apart from the apoptosis-inducing potential of RsAFP2 and HsAFP1, no reports exist on the induction of apoptosis in *C. albicans* by other defensins. Recently, Andrés et al. (2008) demonstrated apoptotic cell death in *C. albicans* by lactoferrin, a protein present in mammalian mucosal secretions with antifungal and antibacterial activity. Other natural peptides/proteins that induce apoptosis in yeast are osmotin (Narasimhan et al., 2001), a truncated derivative of dermaseptin S3 (Morton et al., 2007), yeast pheromone (Pozniakovsky et al., 2005), and yeast killer toxins (Klassen and Meinhardt, 2005).

A considerable part of the identified HsAFP1-tolerance/sensitivity genes encode proteins implicated in vacuolar acidification, vacuolar protein sorting, or vesicular transport. It was recently demonstrated that yeast hampered in vacuolar protein sorting or lacking a functional vacuolar H⁺-ATPase shows multi-drug rather than drug-specific sensitivity (Parsons et al., 2004). Therefore, this

functional group may rather represents general sickness. However, it is also reported that the vacuolar H⁺-ATPase in yeast is required for oxidative stress response (Kane, 2007). Consistent with such a role, *vma* mutants are shown to be hypersensitive to multiple forms of oxidative stress and display elevated levels of ROS even in the absence of an exogenous oxidant (Thorpe et al., 2004; Kane, 2007; Milgrom et al., 2007). This would then suggest that an intact vacuolar H⁺-ATPase is required for HsAFP1 tolerance in *S. cerevisiae*.

The fraction of HsAFP1-tolerance/sensitivity genes implicated in gene expression/DNA repair may also represent general stress sensitivity mechanisms. In this respect, yeasts affected in genes involved in DNA synthesis and repair, transcription, and chromatin structure (including ADA/SAGA histone acetyltransferase complexes or the SWI/SNF nucleosome remodeling complex) were previously identified as hypersensitive to a variety of stresses, including oxidative and chemical stress including treatment with the antifungal miconazole (Thorpe et al., 2004; Thevisen et al., 2007b). Several HsAFP1-tolerance genes (*DHH1*, *CCR4*, *LSM1*, *LSM6*, and *KEM1*) have a function in post-translational mRNA regulation and mRNA decay. These data highlight the importance of *de novo* transcription in response to environmental stress, and may indicate that posttranslational gene regulation plays a role in the cellular stress response against HsAFP1.

We further identified HsAFP1-tolerance genes implicated in different mitogen-activated protein kinase (MAPK) pathways. *CDC73*, *CCR4*, *RTF1*, and *SWI4* are HsAFP1-tolerance genes that are implicated in the MAPK cell integrity pathway. The cell integrity pathway plays a key role in maintaining the cell wall integrity in distinct environmental conditions. This pathway is induced in periods of polarized growth and responds to heat, hypo-osmotic shock, cell wall damage, and oxidative stress (Martin et al., 2005). Swi4p is a transcriptional activator in the cell integrity pathway (Gustin et al., 1998). Cdc73p, Ccd4p, and Rtf1p are constituents of the Paf1 complex that is required for full expression of many cell wall biosynthetic genes in the cell integrity pathway (Porter et al., 2002). In this respect, it has to be noted that we also identified several HsAFP1-tolerance genes involved in cell wall maintenance and architecture (*CHS7*, *VPS52*, *MNN11*, *VAN1*, and *GAS1*). Apparently, the MAPK cell integrity pathway is also involved in protection of *S. cerevisiae* to caffeine (Kuranda et al., 2006), caspofungin (Reinoso-Martin et al., 2003), and farnesol (Fairn et al., 2007), and in protection of *F. graminearum* to RsAFP2 and the medicago MsDef1 (Ramamoorthy et al., 2007). Furthermore, we identified one HsAFP1-tolerance gene, *PTC1*, implicated in the osmosensing high osmolarity glycerol (HOG) MAPK pathway. The HOG pathway responds to osmotic stress. In addition, this MAPK pathway has also been shown to respond to heat shock, oxidative stress, and citric acid (Martin et al., 2005). Remarkably, although Ptc1p negatively regulates the HOG MAPK pathway (Warmka et al., 2001), *PTC1* deletion confers HsAFP1 hypersensitivity. Finally, we identified *SCP160* as a HsAFP1-tolerance gene. Scp160p is an RNA-binding G protein effector of the MAPK mating response pathway (Guo et al., 2003). All these findings suggest the involvement of different MAPK stress response pathways in yeast tolerance toward HsAFP1.

Finally, we identified five HsAFP1-tolerance genes implicated in cytoskeletal organization and cytokinesis. Interestingly, decreased actin turnover was previously shown to result in increased

mitochondrial ROS production and apoptosis (Gourlay and Ayscough, 2006). It was recently proposed that actin residues can act as oxidative stress sensors that further regulate cell death in yeast (Farah and Amberg, 2007). Only one study reports on a role for actin cytoskeleton alterations in the mechanism of action of an apoptosis-inducing antifungal compound. In this respect, the antifungal drug jasplakinolide has been shown to cause a block in actin dynamics (Ayscough, 2000) and to induce ROS and apoptosis in *S. cerevisiae* (Gourlay et al., 2004). Whether HsAFP1 induces changes in the actin cytoskeleton organization and whether this process results directly in elevated ROS levels and subsequent apoptosis, needs to be investigated further.

In conclusion, we identified 71 HsAFP1-tolerance genes and 13 HsAFP1-sensitivity genes, which we subdivided in different groups according to their function. In this way, we were able to deduce part of the HsAFP1 antifungal action mechanism.

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